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Research Article

SUBCLINICAL MASTITIS - ITS PREVALENCE, RISK ANALYSIS, AND ASSOCIATION WITH METHICILLIN-RESISTANT *STAPHYLOCOCCUS AUREUS* (MRSA) IN CERTAIN DISTRICTS OF ASSAM, INDIA

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ABSTRACT: This study investigates the epidemiology of subclinical mastitis, focusing on prevalence, risk factors, bacterial pathogens, virulence factors, antimicrobial sensitivity, and detection of methicillin-resistant *Staphylococcus aureus* (MRSA) bacterial isolates in organized and unorganized dairy farms of Kamrup and adjoining districts of Assam. The overall prevalence of bovine subclinical mastitis was found to be 43.81% using the California Mastitis Test (CMT). Risk factor analysis revealed that age-wise, the highest prevalence was noted in the 4-6 years age group (51.95%) and at 4th lactation (60.40%). Significant associations were found between mastitis prevalence and various factors including age, breed, lactation number, stage of lactation, quarter-wise distribution, farm type, management system, and season. A total of 185 bacterial isolates were obtained, predominantly *Staphylococcus* spp. (143), with notable occurrences of *Streptococcus* spp. (22), *Escherichia coli* (14), and *Enterococcus faecalis* (6). *Staphylococcus aureus* emerged as the primary etiological agent, with 81.82% of isolates producing coagulase and 45.45% producing hemolysin, while 54.0% of *Staphylococcus aureus* isolates tested positive for the protein A (spa) gene. Antimicrobial sensitivity testing revealed enrofloxacin, gentamicin, and ceftriaxone as the most effective antibiotics, with penicillin-G demonstrating the least efficacy. Methicillin resistance was detected in 11.96% of *Staphylococcus aureus* isolates phenotypically and in 2.56% genotypically (mecA) revealing the emergence of MRSA which is important from a public health point of view. These findings highlight the prevalence of subclinical mastitis, the predominance of *Staphylococcus aureus* as the causative agent, and the emergence of antimicrobial resistance, which warrants effective control measures and antimicrobial management in dairy farming practices.

Keywords: Assam, Bovine, Epidemiology, Mastitis, *mec*A, MRSA, *spa.*

INTRODUCTION

The livestock sector is the backbone of India's economy, intimately entwined with agriculture and fisheries. Livestock, crucial for the sustenance of impoverished farmers, contributes significantly to food security, economic stability, and household nutrition, yielding an impressive 155.5 million tons of milk annually alongside substantial quantities of eggs and wool. Published reports [1] assert its formidable contribution, constituting 3.46 percent of the GDP and 29.20 percent of the agricultural GDP, supporting approximately 20.5 million livelihoods. However, this vital sector faces persistent threats, notably from infectious diseases such as bovine mastitis, which inflict severe economic repercussions on dairy production. Mastitis, both clinical and subclinical, manifests as a foremost concern, leading to decreased milk yield, premature culling, and elevated veterinary costs [2, 3].

Reports indicate a staggering increase in economic losses due to mastitis, escalating from Rs. 16,072 million to Rs. 71,655 million within 15 years [4]. The

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increasing global demand for milk production has led to a rising incidence of mastitis, a prevalent issue in dairy herds. Notably, most cases are subclinical, lacking visible symptoms but still adversely affecting cow health and overall productivity [5]. The multifactorial nature of mastitis implicates various factors such as seasonality, breed, hygiene, and management practices. Moreover, the emergence of antimicrobial resistance among mastitis pathogens exacerbates the challenge, rendering conventional antibiotic treatments increasingly ineffective. Another alarming aspect of mastitis is the increased risk of transmission of communicable diseases such as tuberculosis, brucellosis, staphylococcal toxemia, septic sore throat, gastroenteritis, etc. [3, 6] along with antibiotic resistance genes [7].

This present study aims to investigate the prevalence and characteristics of subclinical mastitis in both organized and unorganized dairy farms in Kamrup (Metro) and adjacent districts. Through isolation and identification of key bacterial pathogens from mastitic milk samples, we seek to elucidate the virulence factors of coagulase-positive staphylococci (CPS) using phenotypic and genotypic methods. Additionally, we aim to analyze the antimicrobial susceptibility patterns of the isolates. By addressing these objectives, our study endeavors to enhance our understanding of mastitis dynamics and to formulate control strategies for its effective management at the field level.

MATERIALS AND METHODS

Study area and sample collection

Milk samples for the present study were collected from all the quarters of every lactating cow from organized and unorganized dairy farms in Kamrup (Metro) and adjoining *viz.* Kamrup (Rural), Marigaon, Nagaon, Nalbari, Barpeta, and Darrang districts to screen for subclinical mastitis. The sampling details are provided in Tables No. 2, 3, and 5. Other relevant epidemiological data and attributes were also recorded at the time of collection of samples. Classification of the organized and unorganized dairy farms was done according to the criteria reported earlier [8] where the organized farm is observed with certain facilities like adequate clean water supply, proper ventilation, drainage, and bedding whereas, the absence of all these facilities along with poor housing design is categorized under unorganized dairy farm. Before collection of milk samples, the udder was thoroughly cleaned and teat orifices were rubbed with 70% ethanol. Milk samples from all the quarters were screened for subclinical mastitis using the DeLaval California mastitis test (CMT) kit as depicted in Fig. 1 (a and b) following the manufacturer's instructions.

Ethical permission

The present study was approved by the Institutional Animal Ethics Committee (IAEC), College of Veterinary Science, Assam Agricultural University (AAU), Khanapara, Guwahati, Assam, India.

Identification and isolation of pathogens associated with mastitis

All the media required for isolation of *Staphylococcus* spp., *Streptococcus* spp., and *Escherichia coli* were prepared as published reports [9]. Milk samples on the same day of receipt, were directly streaked onto blood agar plates (sterile 5% defibrinated sheep blood agar) for primary isolation of *Staphylococcus* spp., *Streptococcus* spp., and *E. coli* as per the standard method [10]. The plates were incubated aerobically at 37°C for 24-48 hours and were examined for the presence of growth of the desired organisms. After initial growth in blood agar, staining reaction, colony morphology, and hemolysin production were recorded. From each plate, well-separated colonies were picked up and subcultured onto appropriate media for purification and preliminary characterization. Isolated colonies of Gram-positive cocci suspected for *Staphylococcus* spp were sub-cultured separately on Mannitol Salt Agar (HiMedia laboratories) and Baird-Parker agar (HiMedia laboratories) and *Streptococcus* spp. on Edward's medium (HiMedia laboratories) whereas, Gram-negative bacilli suspected for *E. coli* were sub cultured separately on MacConkey's Lactose Agar (MLA) and Eosin Methylene Blue Agar (HiMedia laboratories) and incubated at 37°C for 24 hours under aerobic conditions [Fig. 2 (a-g)]. Post purification the suspected colonies were streaked onto Nutrient Agar slants and incubated at 37°C for 24 hours. Culture slants were then sealed with molten paraffin and maintained at 4°C in the refrigerator and used whenever necessary.

Biochemical characteristic

Preliminary identification of *Staphylococcus* spp. was performed by the tube coagulase test and *Streptococcus* spp. by CAMP reaction [11,12]. Further, testing of the isolates was carried out using the commercially available biochemical kits (HiStaph, HiStrep and HiIMViC) as per manufacturer protocol (HiMedia laboratories).

Antimicrobial susceptibility

Antimicrobial susceptibility patterns of all isolates recovered during the present study from mastitic milk samples were determined against 18 antimicrobial agents by using the disc diffusion method on Mueller-Hinton agar (HiMedia laboratories) as per the recommendation of the Clinical Laboratory Standard Institute, 2018 [46]. The details of the antibiotics (HiMedia laboratories) used for the antibiogram assay were Penicillin-G (P)-10µg, Oxytetracycline (O)-30 µg, Tetracycline (T)-30 µg, Ampicillin (Amp)-10µg, Enrofloxacin (Ex)-10µg, Gentamicin (GEN)-10µg, Ciprofloxacin (CIP)-5µg, Streptomycin (S)-10µg, Cloxacillin (COX)-30 µg, Amoxycillin (Am)-30µg, Neomycin(N)-30µg, Methicillin(MET)-5µg, Colistin(CL)-10µg, Ceftriaxone(CIR)-30µg, Cefixime (CFM)-5µg, Cefotaxime (CTX)-10µg, Amoxyclav (AMC)-30µg, Ceftazidime (CAZ)-30µg.

Phenotypic and genotypic virulence factors of *Staphylococcus* spp.

Phenotypic virulence factors of *Staphylococcus* spp. were determined by performing hemolysins and coagulase production tests. For studying genotypic virulence factor, the gene coding for staphylococcal protein A (spa) was targeted with a product size of 920 bp by using polymerase chain reaction (PCR). Primer sequence spa-F (5'CACCTGCTGCAAATGCTGCG3') and spa-R (5'GGCTTGTTGTTG TCTTCCTC3') were employed as per the published report [13]. The thermal cycling condition for amplification is depicted in Table 1a.

Detection of methicillin-resistant *Staphylococcus aureus* (MRSA)

Two well-isolated colonies of *Staphylococcus aureus* on Mannitol Salt Agar were picked up and sub cultured separately on MeReSaHiVeg Agar Base with MeReSa supplement and cefoxitin supplement (HiMedia laboratories Ltd. Mumbai) and incubated at 37°C for 24 hours under aerobic condition for detection of MRSA (Fig. 2h). Further, molecular detection and amplification of mecA gene was carried using primer sequence mecA - F (5'AAAATCGAGTGTAAAAGGTT GGC3') and mecA - R (5'AGTTCTGCAGTAACCGGA TTTGC3') as per report [14]. Thermal cycling conditions for amplification are depicted in the Table 1b. All the positive controls were sourced from the bacterial repository of the Microbiology Department, CVSc. AAU, Khanapara, India.

Statistical analysis

Statistical analysis of the factors affecting the prevalence of subclinical mastitis in cows was done (Chi-square test) by using SPSS (Statistical Package for Social Science), Version 14.

RESULTS AND DISCUSSION

Overall prevalence

In the present study, a total of 388 animals were screened for subclinical mastitis using the California Mastitis Test (CMT). Of these, 170 cows were found positive for subclinical mastitis. The overall prevalence was found to be 43.81%. (Table 2). The highest prevalence of subclinical mastitis was found in Darrang district (53.33%) and lowest in Kamrup (Rural) (21.57%). Similar observation on the prevalence of subclinical mastitis in cows, ranging between 44.0% and 59.70% was also made by several workers [15, 16].In a meta-analysis of studies, the prevalence of subclinical mastitis in dairy cows in India was found to be 46.35% animal-wise and 23.25% quarter-wise. It was also observed that a lot of variation in prevalence estimates existed between studies [17]. On the contrary, other workers reported the prevalence of subclinical mastitis as low as 13.84 to 19.14% [18, 19, 20]. However, higher prevalence rates of 71.0 to 75.6 % for subclinical mastitis were also documented [21, 22]. In the present study, the prevalence of subclinical mastitis on a quarter basis was found to be 18.04% which revealed statistically significant differences ($p<0.05$) exist quarter-wise (Table 2). Similarly, a lower prevalence of subclinical mastitis on a quarter basis ranging from 12.38 to 26.9 %was also reported earlier [23, 24]. On the other hand, the prevalence as high as 44.17% was also reported earlier [25]. These variations in the prevalence of subclinical mastitis reported might be attributed to the differences in management systems adopted, the agro-climatic condition of the study areas, and the lack of awareness among farmers regarding the principles of farm and self-hygiene. Apart from these factors, the variation might also be attributable to the invisible and silent nature of subclinical mastitis.

Management and host factors

Breed-wise prevalence

The prevalence of bovine subclinical mastitis was highest in the Jersey cross (56.31%) followed by the Holstein-Friesian cross (39.13) and lowest in the indigenous cattle breed (13.43%). Statistically, the chi-

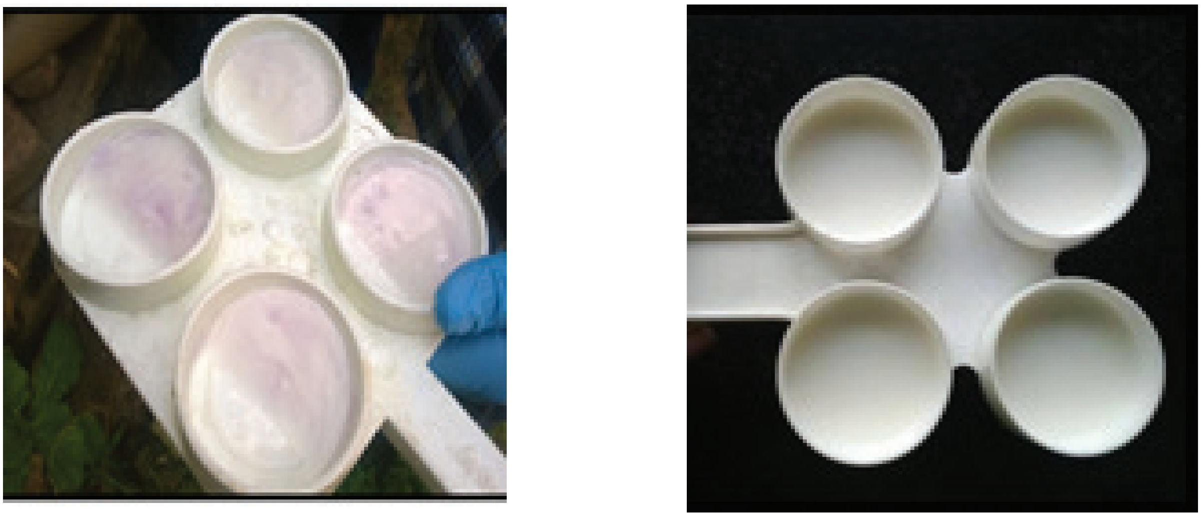
Table 1. Thermal cycling conditions for amplification of staphylococcal protein A (spa) and mecA gene.

Steps	Initial denaturation	Denaturation	Annealing	Extension	Final extension	Hold
a) Staphylococcal protein A (*spa*) at IgG binding region						
Temperature	95°C	94°C	66°C	72°C	72°C	4°C
Duration (minute)	3	1	1	1	10	
No. of cycles	1		30		1	
b) *mecA* gene						
Temperature	95°C	94°C	55°C	72°C	72°C	4°C
Duration (minute)	3	1	1	1	10	
No. of cycles	1		30		1	

Table 2. Prevalence of bovine subclinical mastitis in Kamrup and adjoining districts of Assam.

Districts	Animal-wise prevalence				Quarter-wise prevalence				No. of samples collected for bacteriological examination
	No. of the animals examined	No of animals Positive in CMT	%	Chi-square value	No. of quarters examined	No. of quarters positive in CMT	%	Chi-square value	
Kamrup(M)	70	36	51.43		280	98	35.00		78
Kamrup(R)	51	11	21.57		204	21	10.29		15
Marigaon	52	25	48.08		208	24	11.54		24
Nagaon	55	24	43.64		220	28	12.73		16
Nalbari	48	20	41.67		192	40	20.83		20
Barpeta	52	22	42.31		208	32	15.38		24
Darrang	60	32	53.33		240	37	15.42		23
TOTAL	388	170	43.81	14.632*	1552	280	18.04	76.014**	200

*Significant (p<0.05) ; **Highly significant (p<0.01)



(a) (b)

Fig 1 (a-b). Screening for subclinical mastitis by California mastitis test using mastoid paddle.
[a: California mastitis test (Positive); b: California mastitis test (Negative)].

Table 3. Prevalence of bovine subclinical mastitis in relation to various epidemiological attributes.

Breed/genetic group	Animals tested	Positive	Prevalence (%)	Chi-square value
1. Breed-wise				
Jersey cross	206	116	56.31	
HF cross	115	45	39.13	
Local	67	9	13.43	39.21417**
Total	388	170	43.81	
2. Age-wise (years)				
2-4	71	23	32.39	
4-6	179	93	51.95	
6-8	102	45	44.12	13.76078**
8 and onwards	36	9	25.00	
Total	388	170	43.81	
3. Number of lactations				
1st lactation	29	8	27.59	
2nd lactation	42	14	33.33	
3rd lactation	79	34	43.04	
4th lactation	101	61	60.40	
5th lactation	58	26	44.83	
6th lactation	43	18	41.86	21.54404**
7th lactation	23	6	26.09	
8th lactation	13	3	23.08	
Total	388	170	43.81	
4.Stage of lactation				
Early (1st to 3rd month)	112	67	59.82	
Mid (4th to 6th month)	183	83	45.35	
Late (above 6 month)	93	20	21.50	30.63568**
Total	388	170	43.81	

**Highly significant ($p<0.01$)

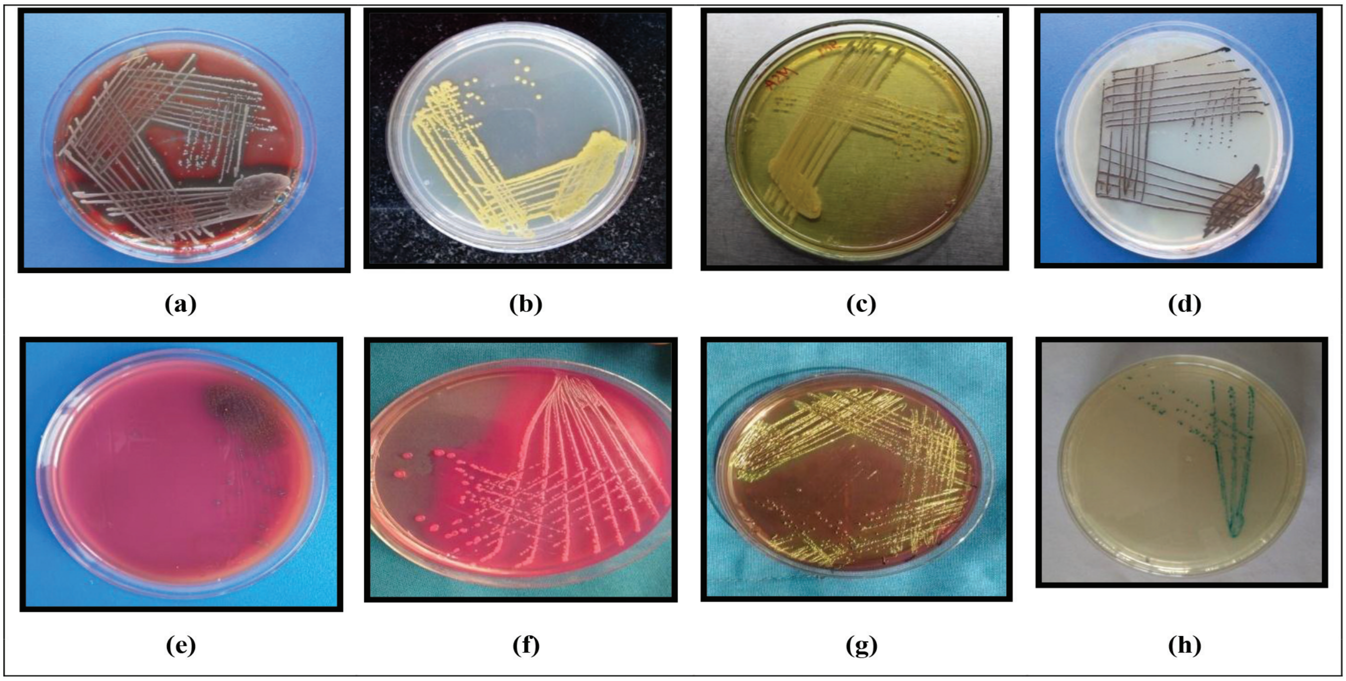


Fig. 2(a-h). Culture and identification of bacteria in various growth media. [a: *Staphylococcus* spp. on Sheep blood Agar; b: *Staphylococcus* spp. on Nutrient agar; c: *Staphylococcus* spp. on Mannitol salt agar; d: *Staphylococcus* spp. on Baird-Parker agar; e: *Streptococcuss* spp. on Edward's medium; f: *E. coli* on MacConkey's Lactose Agar; g: *E. coli* on Eosin Methylene Blue agar; h: Growth of methicillin resistant *Staphylococcus aureus* (MRSA) on MeReSaHiVeg agar base (Bluish-green coloured colonies)].

Table 4. Prevalence of bovine subclinical mastitis in relation to affected quarters.

1. Quarter-wise distribution

Affected quarters	Right fore quarter	Right hind quarter	Left fore quarter	Left hind quarter
280	20 (7.14 %)	87 (31.07 %)	45 (16.07%)	128 (45.71%)
Chi-square value	96.82857**			

2. Number of quarters affected with involvement

Affected quarters	One quarter	Two quarters	Three quarters	Four quarters
170	100 (58.82%)	30 (17.65%)	12 (7.06%)	28 (16.47%)
Chi-square value	108.3059**			

**Highly significant (p<0.01).

Table 5. Prevalence of bovine subclinical mastitis about management system and season.

1. Farm type

Farm types		Animals tested	Positive	Prevalence (%)	Chi-square value
Organized farm		78	25	32.05	
Unorganized farm		310	145	46.78	5.487*
Total		388	170	43.81	

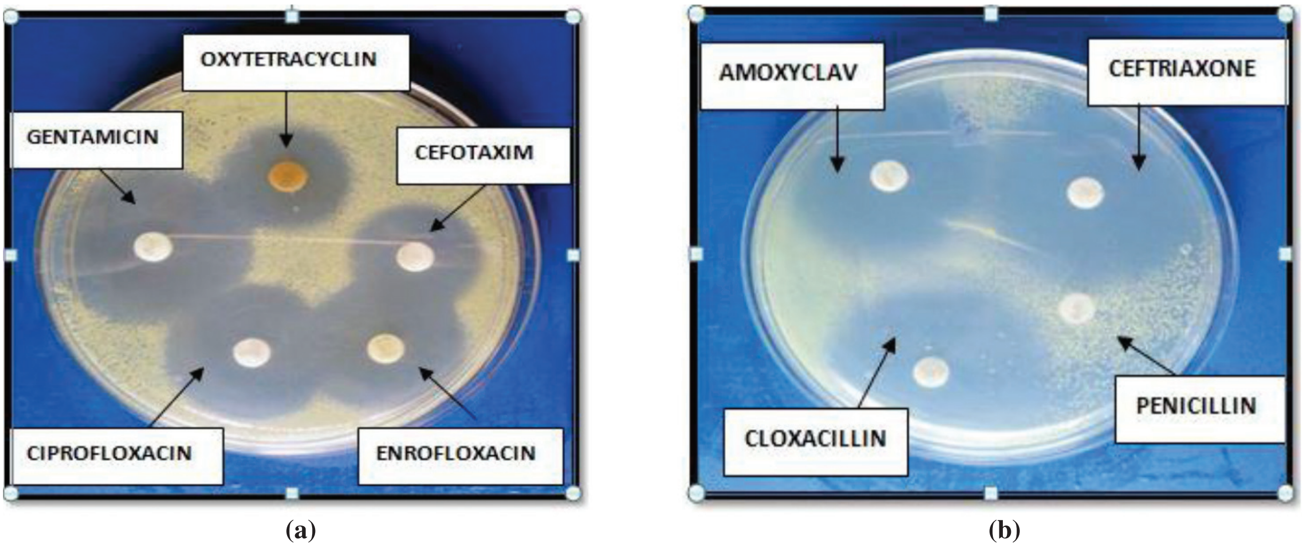
2. Management system

Management system		Animals tested	Positive	Prevalence (%)	Chi-square value
Housing system	Conventional barns	310	149	48.06	11.31493**
	Loose housing	78	21	26.92	
Hygienic condition	Good	168	58	34.52	10.38882**
	Poor	220	112	50.91	

3 Season-wise

Season		Animals tested	Positive	Prevalence (%)	Chi-square value
Spring	(March-May)	93	37	39.78	
Summer	(Jun-August)	105	46	43.81	
Autumn	(September-November)	78	21	26.92	20.04672**
Winter	(December-February)	112	66	58.93	

*Significant (p<0.05) ; **Highly significant.



(a) **(b)**

Fig. 3(a-b). Antimicrobial sensitivity pattern of *Staphylococcus aureus* on Mueller Hinton Agar plates.

Table 6. Isolation of pathogenic bacteria from bovine subclinical mastitis.

No of samples positive/tested	Types of bacteria isolated	No. of isolates obtained
133/200(66.50%)	*Staphylococcus* spp.	133
12/200(6.0%)	*Streptococcus* spp.	12
6/200 (3.0%)	*Enterococcus faecalis*	6
14/200(7.0%)	*Escherichia coli*	14
10/200 (5.0%)	Mixed culture	20
175/200*		185

*Mixed cultures of *Staphylococcus* spp. and *Streptococcus* spp. were yield by 10 out 200 samples.

Table 7. Isolation of pathogenic bacteria from bovine subclinical mastitis cases.

Bacterial species isolated	No. of isolates	Percentage
Staphylococcus aureus	117	63.24
Staphylococcus epidermidis	11	5.94
Staphylococcus auricularis	6	3.24
Staphylococcus capitis sub spp. *capitis*	2	1.08
Staphylococcus hominis	1	0.54
Staphylococcus chromogenes	6	3.24
Streptococcus dysgalactiae	13	7.03
Streptococcus uberis	5	2.70
Streptococcus pyogenes	4	2.16
Enterococcus faecalis	6	3.24
Escherichia coli	14	7.57
Total number of isolates	185	

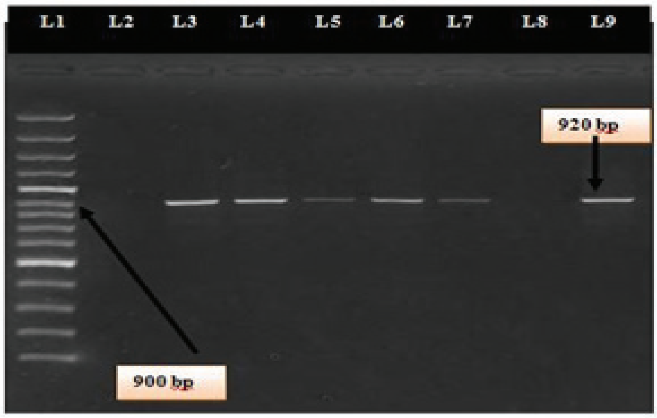


Fig. 4. PCR product of spa (IgG region) [Lane 1 = 100 bp ladder, Lane 2 = non-template control, Lane 3 to 7 = sample, Lane 8 = Blank, Lane 9 = Positive control].

Table 8(a). Antibiogram of microorganisms isolated from subclinical mastitic milk samples.

Species	Isolates tested	No. of isolates sensitive to various antimicrobial disc (in percentage)								
		P	O	T	Amp	Ex	GEN	CIP	S	COX
Staphylococcus aureus (CPS)	117	35 (29.91%)	114 (97.43%)	114 (97.43%)	50 (42.73%)	117 (100%)	117 (100%)	114 (97.43%)	80 (68.38%)	98 (83.76%)
Coagulase -ve *Staphylococci* (CoNS)	26	6 (23.08%)	21 (80.77%)	20 (76.92%)	8 (30.77%)	26 (100%)	22 (84.61%)	22 (84.61%)	16 (61.54%)	11 (42.31%)
Streptococcus spp.	28	11 (39.28%)	22 (78.57%)	22 (78.57%)	20 (71.43%)	28 (100%)	23 (100%)	23 (82.14%)	18 (64.28)	22 (78.57%)
Escherichia coli	14	2 (14.28%)	8 (57.14%)	8 (57.14%)	2 (14.82%)	14 (100%)	14 (100%)	12 (85.71%)	12 (85.71)	2 (14.28%)

Table 8(b). Antibiogram of microorganisms isolated from subclinical mastitic milk samples.

Species	Isolates tested	No. of isolates sensitive to various antimicrobial disc (in percentage)								
		MET	Am	N	CL	CTR	CFM	CTX	AMC	CAZ
Staphylococcus aureus (CPS)	117	105 (89.74%)	98 (83.76%)	114 (97.43%)	-	114 (97.43%)	57 (48.72%)	98 (83.76%)	98 (83.76%)	-
Coagulase -ve *Staphylococci* (CoNS)	26	-	8 (30.77%)	18 (69.23%)	-	26 (100%)	18 (69.23%)	24 (92.31%)	24 (92.31%)	-
Streptococcus spp.	28	-	22 (78.57%)	16 (57.14%)	-	28 (100%)	23 (82.14%)	23 (82.14%)	23 (82.14%)	-
Escherichia coli	14	-	-	8 (57.14%)	10 (71.43%)	12 (85.71%)	12 (85.71%)	12 (85.71%)	8 (57.14%)	10 (71.43%)

Table 9. Phenotypic detection of virulence factors.

***Staphylococcus* spp. isolated and tested**	**No. of isolates +ve for virulence factor**	
	Coagulase +ve	Haemolysi +ve
143	117 (81.82%)	65(45.45%)

Table 10. Detection of gene encoding staphylococcal protein A (spa) at IgG binding region in coagulase-positive *Staphylococci* (CPS).

No. of coagulase +ve staphylococci tested	**No. of isolates positive for gene encoding Protein A (*spa*)**	**Percentage**
117	64	54.70%

Table 11. Prevalence of Methicillin resistant *Staphylococcus aureus* (MRSA)

No. *Staphylococcus aureus* isolates tested	**Phenotypically +ve**	**Genotypically +ve (*mecA*)**
117	14 (11.96%)	3 (2.56%)

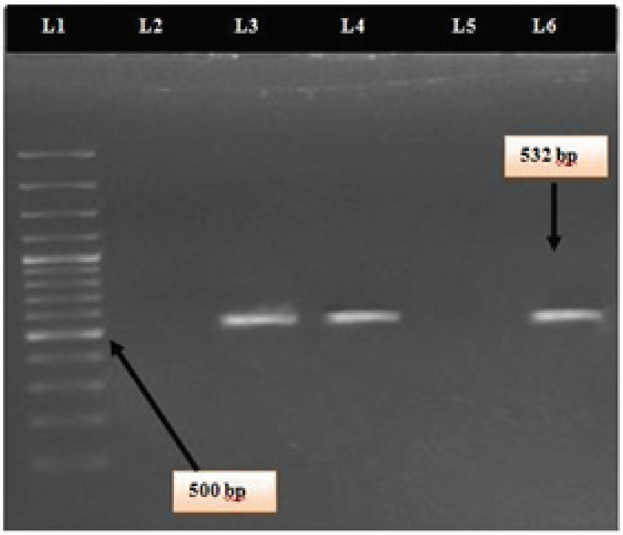


Fig. 5. PCR product of mecA (Lane 1 = 100bp ladder, Lane 2 = non-template control, Lane 3 & 4= Sample, Lane 5 = Blank, Lane 6 = Positive control).

square test revealed that there was a significant correlation (p<0.01) between the breed and with prevalence of bovine subclinical mastitis (Table 3.1). This observation is by previous reports [23, 24]. This observation may be explained based on the fact that cross-breed cows are high-yielders and are more susceptible to mastitis than Indigenous cows who are low milk producers. Also, the difference in the anatomical structure of cross-breed cows exists where their udder is larger and heavier, which makes the animal more prone to lie down all the time thus increasing the chances of ascending infection from the contaminated floor.

Age and lactation

Prevalence of subclinical mastitis was highest (51.95%) in cows of the 4 to 6 years age group and highest (60.40%) in cows at the 4th lactation period (Table 3.2 and Table 3.3). Statistically, the chi-square test revealed that there was a significant relation in the prevalence of bovine subclinical mastitis with age group (p<0.01) and with the lactation number (p<0.01). In agreement with these findings, previous studies [8, 26, 27] have recorded the highest prevalence in similar age groups and lactation periods. Also, it was observed that cows in their early stage of lactation (59.82%) were more commonly affected and revealed a highly significant (p<0.01) association between the stage of lactation and the prevalence of bovine subclinical mastitis. The present observation is in agreement with other workers [18, 28, 29]. This association can be related to the underlying stress of recent parturition, delayed diapedesis of neutrophils into the mammary tissue, and low antioxidant defense during this period.

Quarter-wise affection

The present study revealed that the left hind quarter (45.71%) and right hind quarters (31.07%) were mostly affected as compared to the other two quarters and it was also observed that single-quarter involvement (58.82%) was more common in bovine subclinical mastitis. Statistically, there was a significant (p<0.01) correlation between quarter affection and with prevalence of subclinical mastitis (Table 4.1and 4.2).In similar quarter prevalence studies of subclinical mastitis, the researchers reported the highest incidence in a single quarter followed by two and four [8]. Also highest ratio of infection (50.5%) was recorded in the right hind (RH) teats followed by the left hind (LH) (43.9%) [18].

Farm type and management

The prevalence of subclinical mastitis was found to be higher in unorganized farms (46.78%) in comparison to the organized farms (32.05%) and also recorded higher in conventional barns (48.06%) and poor hygienically reared animals (50.91%). Statistically, the chi-square test revealed that there was a significant correlation between farm type (p<0.05) and housing system and hygienic condition (p<0.01) with the prevalence of bovine subclinical mastitis as depicted in Table 5.1 and Table 5.2.

Published studies [8, 30] also recorded a higher prevalence of subclinical mastitis in unorganized than organized farms and in conventional farms with poor hygiene [8, 23].

Season

The temporal study revealed the highest prevalence of subclinical mastitis was recorded in winter (58.93%) and lowest in autumn (26.92%) which might be due to the higher rate of calving and peak milk production in winter that put the teat sphincter under stress leading to increased chance of ascending infection. Also, statistically, there was a significant ($p<0.01$) association of season with prevalence of bovine subclinical mastitis (Table 5.3). In a similar study, the incidence of bovine mastitis was found to be highest in winter followed by summer, and least in the rainy season [31].

Isolation of bacteria

A total of 185 isolates of bacteria were obtained from the 200 CMT-positive milk samples, collected from the representative animals of the herds screened as depicted in Table 6. Among the various species of bacteria isolated, *Staphylococcus aureus* was found to be the chief etiological agent of subclinical mastitis in cows. Similar findings were reported by other researchers [22, 32, 33] who also stated that *S. aureus* is one of the chief etiological agents for causing subclinical mastitis. The higher prevalence of *S. aureus* might be attributed to the fact that the principal site of predilection for this microbe is the skin of the udder and milk of the infected gland with the capacity to penetrate the tissue producing deep-seated foci. Also, *S. aureus* is a contagious organism. In addition to *S. aureus*, *Streptococcus* spp., and *Escherichia coli* were found to be the second and the third major causative agent for subclinical mastitis respectively. The details are provided in Table 7. Similar observations were also recorded earlier [34, 35]. The reason is attributable to the fact that *Streptococcus* spp. are the commensal organisms in the milker's hand as well as the skin of the udder which makes the organism more prone to enter the udder through the teat canal while hand milking. Similarly, fecal contamination of the farm floor or from an unhygienic immediate environment increases the chance of *E. coli* infection.

Antimicrobial susceptibility

In the present study, all the isolates including *Staphylococcus* spp., *Streptococcus* spp., *E. coli*, and *Enterococcus faecalis* recovered from subclinical mastitis exhibited a high degree of sensitivity to enrofloxacin, gentamicin, and ceftriaxone followed by oxytetracycline, ciprofloxacin and amoxyclav and less sensitive towards penicillin-G [Table 8 (a and b) and Fig. 3(a-b)]. This observation was more or less similar to the observation made earlier. While studying the pattern of antibiotic resistance in dairy cows of Tamil Nadu, it was reported that *E. coli*, *S. aureus*, and MRSA organisms showed more sensitivity to enrofloxacin, amoxicillin + sulbactam, gentamicin, and ceftriaxone and had highest resistant to penicillin [28]. In another study, the *in-vitro* antibiogram of mastitis pathogens revealed higher sensitivity for enrofloxacin, a combination of cefalexin + kanamycin and amoxicillin + clavulanic acid. Most of the pathogens exhibited the highest resistance against penicillin [36]. The probable reason behind this could be that enrofloxacin, gentamicin, and ceftriaxone are comparatively new and less commonly used in the prophylaxis as well as treatment of mastitis whereas less sensitivity to penicillin-G might be due to the extensive and injudicious use of the same in intra-mammary preparation for the treatment of udder infection. Antimicrobial agents are routinely used for the treatment of dairy cattle affected with subclinical mastitis. Excessive use of antimicrobial agents has increased the number of resistant microbes globally. The injudicious use of antimicrobial drugs, self-medication, frequent switching of antibiotics, and indecorous treatment given by non-veterinarians and quacks in recent times, mostly contribute to the increase in antimicrobial resistance without improving the outcome of treatment [37, 38]. Several workers have reported resistance to bovine subclinical mastitis pathogens against different antimicrobial drugs.

Phenotypic and genotypic virulence factor of *Staphylococcus* species

In the present study, among the *Staphylococcus* spp. 117(81.82%) isolates produced coagulase and 65 (45.45%) produced hemolysin (Table 9). Similar observations were also opined in earlier studies [39, 40, 41]. However, hemolysin production by *Staphylococcus* spp. was found to be less in comparison to coagulase, which might be due to the differences in the strains, culture conditions, or preparation and processing of samples [41]. Subsequently, 64 (54.0%) isolates found to be coagulase-positive *Staphylococci* (CPS) out of 117 *Staphylococcus* spp. were detected positive for gene encoding protein A (spa) at IgG binding region and the isolates yielded an amplified product of 920bp (Table 10 and Fig. 4). Earlier while working on *S. aureus* isolates from mastitic milk, Akineden *et al.* (2001) reported that the PCR amplification of the gene encoding the IgG binding region of protein A revealed a size of 920 bp for most of the isolates investigated [39].

Methicillin-resistant *Staphylococcus aureus* (MRSA)

In the present study, methicillin resistance in *Staphylococcus aureus* (MRSA) was detected in 14 isolates (11.96%) phenotypically by using HiCHROME agar and 3 isolates (2.56%) genotypically by amplification of the genes encoding mecA (Table 11 and Fig. 5). The present finding is consistent with that of earlier report [42]. Thus, 11 isolates of *Staphylococcus aureus*, phenotypically resistant to methicillin in this study did not carry the mecA gene. This discrepancy in the correlation between the mecA gene and phenotypic methicillin resistance has been reported earlier also [42, 43]. The possible reasons for this might be related to the poor expression of mecA genes due to the production of methicillinase or may be due to overproduction of β-lactamase. Methicillin resistance *Staphylococcus aureus* (MRSA) has recently emerged as a serious life-threatening infective agent, which does not respond to a lot of antimicrobial treatments [44, 45]. Methicillin resistance *Staphylococcus aureus* (MRSA) is any strain of *Staphylococcus aureus* that has developed, through horizontal gene (mecA) transfer, natural selection, and multiple drug resistance to β-lactam antibiotics. The first report of mecA in MRSA from bovine milk was reported in 2003 [44]. The presence of MRSA in bovine mastitic milk can cause an increase in the risk of transmission of the mecA gene to other animals as well as the human population [45].

CONCLUSION

This study provides comprehensive insights into the epidemiology of bovine subclinical mastitis in dairy farms across Kamrup and adjoining districts, revealing an overall prevalence of 43.81%. The highest prevalence was observed in the Darrang district (53.33%), while Kamrup (Rural) had the lowest rate (21.57%). *Staphylococcus aureus* was identified as the predominant pathogen, followed by *Streptococcus* spp. and *Escherichia coli.* Antimicrobial susceptibility testing showed high sensitivity to enrofloxacin, gentamicin, and ceftriaxone, but notable resistance to penicillin-G. Methicillin-resistant *Staphylococcus aureus* (MRSA) was found in 11.96% of isolates phenotypically and 2.56% genotypically, highlighting an urgent need for effective antimicrobial management. The study also noted significant variations in mastitis prevalence related to breed, age, lactation stage, and farm management practices. These findings underscore the urgent need for effective control measures and antimicrobial management in dairy farming practices to mitigate economic losses and public health risks associated with mastitis.

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